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PRINCIPAL INVESTIGATOR: Celina G. Kleer, M.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, MI 48109-1274

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Annual Report for Award Number: DAMD17-02-1-0490 Career Development Award
(Reporting period 17 April 2004- 16 April 2005)

Introduction

This is the third annual report for a project that aims at understanding the clinical utility of RhoC-GTPase and WISP3 proteins in breast cancer patients. These two genes were identified as key genetic determinants of inflammatory breast cancer (IBC). We believe that RhoC GTPase and WISP3 act in concert to determine a highly metastatic breast cancer phenotype, and that they may help identify which invasive breast carcinomas are aggressive from the outset and treat them more appropriately before the development of metastases. Specifically, we aim to determine whether detection of RhoC GTPase and WISP3 proteins in breast cancer tissue samples can identify aggressive tumors. A second goal of our award is to determine the effect of farnesyl transferase inhibitors (FTIs) in RhoC overexpressing xenografts. This award resulted in significant contributions to advance the knowledge of IBC and generate novel hypotheses.

Body

The major advances on this project have been to develop tissue microarrays (TMAs) using invasive breast carcinomas from 236 patients treated at our institution with over 10 years of follow up information, linked to a clinical database, and to better understand the functional significance of the WISP3 gene in Inflammatory Breast Cancer (IBC), to determine the prognostic utility of RhoC protein expression. Furthermore, we have developed and optimized key reagents to test the expression levels of WISP3 in breast cancer tissue samples. We have published several peer-reviewed publications based on these results. Below are brief descriptions of key accomplishments according to the approved statement of work (SOW):

Task 1. To determine whether the concordant alterations of RhoC-GTPase over-expression and WISP3 loss are prognostic indicators and predictors of survival in breast cancer patients. Months 1-24.

a. Identify and retrieve the breast cancer tissue blocks and slides (489 cases total). Months 1-6.
b. Histopathologic study of the cases and selection of adequate tumor areas to construct the tissue microarrays. Categorize the breast cancers according to stage. Months 6-9.
c. Construction of the tissue microarrays, one containing 400 breast cancers of all anatomic stages and the other containing 89 cases of locally advanced breast cancer. Months 9-15.
d. Immunohistochemical analysis for RhoC-GTPase and WISP3 proteins, and other markers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis). Months 16-19.
e. Interpretation and grading of the immunohistochemical studies and statistical analyses. Months 20-24.

Task 1

a. Identify and retrieve the breast cancer tissue blocks and slides.

By performing a computerized search of the breast cancer database at the Department of Pathology, University of Michigan, using the words "breast" and "cancer" and "breast" and "carcinoma" from

years 1987-1991. We identified 385 consecutive invasive breast cancer patients. Of the 385 cases, 236 cases were available for study. The reasons for this were: 1. unavailability of tissue slides or blocks, and 2. primary resection performed at a referring institution.
I n addition, 60 cases of locally advanced breast cancer, of which 30 are inflammatory breast cancers, and 30 are stage matched, non-inflammatory breast cancer were identified from the pathology files.

b. Histopathologic study of the cases and selection of adequate tumor areas to construct the tissue microarrays. Categorize the breast cancers according to stage.

The P.I. reviewed all the hematoxylin and eosin stained sections from all these cases and annotated the pathologic characteristics of each tumor using the following template:

Summary for Invasive Carcinomas.

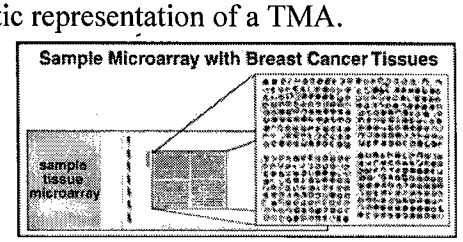
Greatest dimension of invasive carcinoma (microscopic):						**cm**
Involvement of surgical margin:		**Positive (at ink)**				**Close (<= 0.2 cm)**
		Negative (>0.2 cm)				
If margin positive:			**Single focus**			**Multiple foci**
If margin close:			**Single focus**			**Multiple foci**
Histopathological grade (Elton and Ellis):			**1**	**2**	**3**	
Positive lymph nodes /total lymph nodes:				/		
Highest axillary node positive:			**Yes**		**No**	**N/A**
Extranodal extension:			**Yes**		**No**	**N/A**
Extensive DCIS:			**Yes**		**No**	**N/A**
DCIS > 25% of tumor:			**Yes**		**No**	
Extratumoral DCIS:			**Yes**		**No**	
Microcalcifications:	**None**		**within inv/DCIS**			**within benign ducts**
Hormonal receptors:	ER:	**POS**	**NEG**		PR:	**POS** **NEG**
Her2neu overexpression:		**POS (2+**		**3+)**	**NEG**	
T	**N**	**M**				

Development of a breast cancer database

We developed a relational database in Microsoft Access to store the pathological and clinical information. The idea behind this decision was to be able to link the results of the TMA scoring with the patient pathological and clinical information. Clinical and treatment information was extracted by chart review, performed with IRB approval. The P.I. was involved in all steps of the database design and development, and learned how to perform database queries.

c. Construction of the tissue microarrays

We have constructed four high density tissue microarrays (TMAs) that will enable us to characterize WISP3 and RhoC expression in a wide range of normal breast and breast disease, and to study associations between expression of these proteins and patient outcome. The figure below is a schematic representation of a TMA.



In order to construct the tissue arrays, the P.I. reviewed all cases histologically and selected the areas to array. At least three different areas of the tumors were

selected and at least three tissue cores (0.6 mm in diameter) were sampled from each donor block. TMAs are assembled using the manual tissue puncher/array (Beecher Instruments). This instrument consists of thin-walled stainless steel needles with an inner diameter of approximately 600 μm and stylet used to transfer and empty the needle contents. The assembly is held in an X-Y position guide that is manually adjusted by digital micrometers. Small biopsies are retrieved from selected regions of donor tissue and are precisely arrayed in a new paraffin block. Cores are inserted into a 45 x 20 x 12 mm recipient block and spaced at a distance of 0.8 mm apart.

d. Immunohistochemical analysis for RhoC-GTPase and WISP3 proteins, and other markers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis).

We have optimized the conditions for immunohistochemistry for the anti-RhoC antibody and applied it to the constructed TMAs successfully. We used 1:400 dilution of antibody incubated overnight, and microwave antigen retrieval. Below are examples of tissues stained using RhoC antibody:

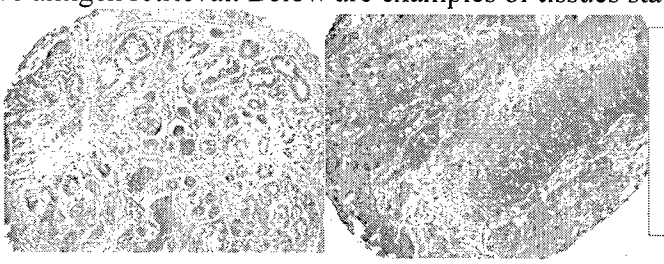


Figure 2. Examples of a tissue microarray element with a hyperplastic benign breast lobule staining weakly for RhoC (left), and an invasive carcinoma, staining strongly for RhoC (right).

We have worked closely with Covance in developing two antigenic peptides and immunizing rabbits to obtain polyclonal antibodies against WISP3. The following peptides were synthesized and polyclonal antibodies were obtained:

Ac-CSGAKGGKKDSDQSN-CONH2
Ac-CPEGRPGEVSDAPQRKQ-CONH2.

After evaluating 4 different anti-WISP3 antibodies, we selected the one that worked better for Western blot and gave a specific band (shown below).

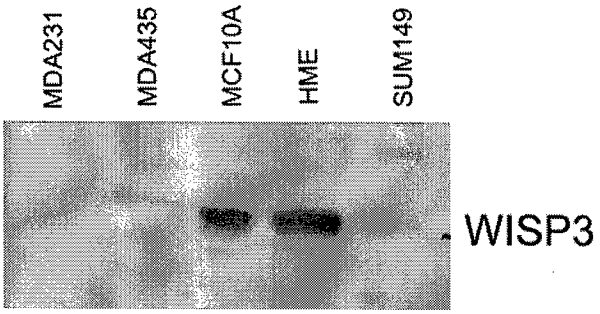


Figure 3. Western immunoblot of cell lysates of five different breast cancer cell lines (MDA231, MDA435, SUM149), and HPV immortalized human mammary epithelial cells (HME), and spontaneously immortalized human mammary epithelial cells (MCF10A). WISP3 protein is expressed in normal cells, and its expression decreases in breast cancer cells.

We have optimized the conditions for the anti-WISP3 antibody for immunohistochemistry and we have applied it to the TMAs successfully. We use the antibody at 1:100 dilution, with 60 minutes incubation and microwave antigen retrieval. Below are examples of the tissues microarray samples stained with anti-WISP3 antibody. We have also stained the TMAs for estrogen receptor, progesterone receptor and HER-2/neu.

e. Interpretation and grading of the immunohistochemical studies and statistical analyses.
I evaluated the immunohistochemistry for RhoC, ER, PR and HER-2/neu in all the TMAs, and with the assistance of Kent Griffith, the biostatistician, have analyzed the results which are shown below. I am in the process of evaluating the immunohistochemistry for WISP3, to explore its clinical relevance. Below is the summary of our RhoC analyses, which are the subject of a manuscript that is in press in Breast Cancer Research and Treatment (see appendix).

We found that RhoC expression increases with breast cancer progression. All samples of normal breast epithelium had negative to weak staining, whereas staining intensity increased in hyperplasia, DCIS, invasive carcinoma, and metastases (Kruskal-Wallis $p<0.001$).
RhoC expression was associated with negative ER expression and worse histologic grade. The table below show the associations between RhoC expression and clinical and pathologic features.
In patients with invasive carcinoma, high RhoC expression was an independent predictor of death from breast cancer, and of local-recurrence free survival. The hazard ratio for local recurrence for patients with high RhoC levels as compared with those with low RhoC levels was 2.37, with a 95% confidence interval of 1.18-4.77 ($p=0.015$), Figure 4.

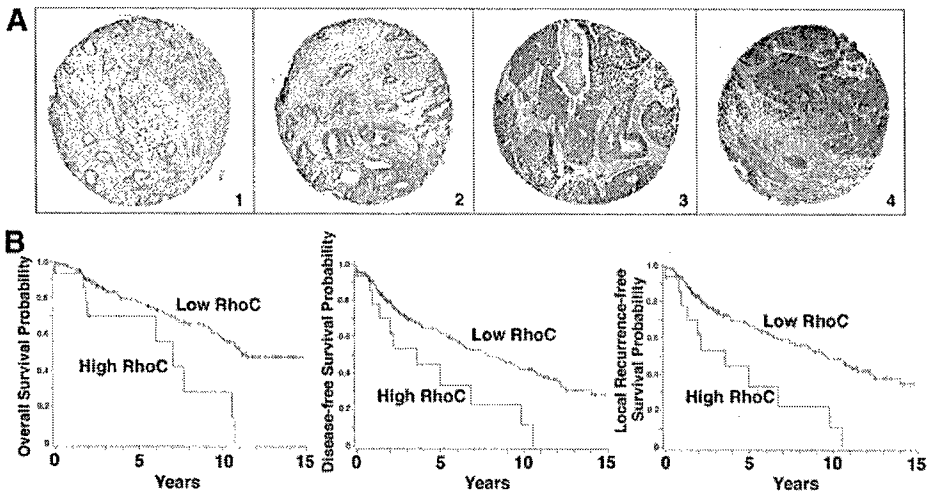


Figure 4. **RhoC protein expression is associated with survival in patients with breast cancer. A.** Tissue microarray elements containing representative invasive carcinomas with negative (1), weak (2), moderate (3), and strong (4) RhoC staining intensities. Original magnification 40x. **B.** High RhoC expression in invasive carcinomas is associated with worse overall, disease-free, and local recurrence-free survival.

Our preliminary studies show that RhoC expression increases with breast cancer progression and RhoC protein levels in tumor tissue, as measured by immunohistochemistry, are strongly associated with survival and local recurrence in patients with breast cancer.This not only extends our initial observations (Kleer et al, Am J Pathol 2002 Feb;160(2):579-84), but also suggests that RhoC may have a role in the local invasiveness and progression of breast carcinoma. Our studies suggest that RhoC protein levels may be first altered in carcinoma *in situ*, the precursor of invasive carcinoma.

Clinically, our retrospective study suggests that RhoC levels may prove useful after further validation, to identify patients with breast cancer that are likely to recur locally.

Task 2. To define the role of Rho-GTPase and WISP3 in the clinical setting as independent predictors of survival in patients with locally advanced breast cancer. Months 24-36.

a. Histopathologic study of 89 cases of locally advanced breast cancer that were previously retrieved
from the pathology files (first 6 months). Selection of adequate areas to construct the tissue microarray. Months 24-27.
b. Development of the tissue microarray, and immunohistochemical analysis of RhoC-GTPase, WISP3 and other biomarkers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis). Months 28-33.
c. Interpretation and grading of the immunohistochemical stains and statistical analyses. Months 33- 36.

Task 2.

a. Histopathologic study of 89 cases of locally advanced breast cancer that were previously Retrieved from the pathology files (first 6 months). Selection of adequate areas to construct the tissue microarray. Months 24-27.
So far, we have identified 60 cases of locally advanced breast cancer, of which 30 are inflammatory breast cancers, and 30 are stage matched, non-inflammatory breast cancer were identified from the pathology files. We evaluated them histologically and chose the areas to construct a TMA
d. Development of the tissue microarray, and immunohistochemical analysis of RhoC-GTPase, WISP3 and other biomarkers (ER, PR, HER2/neu, Ki-67, microvessel density and apoptosis). Months 28-33.
We have constructed a TMA with these tissues, and stained them for RhoC, ER, PR and HER-2/neu. We have stained the TMA for WISP3 as well and are in the process of evaluating the immunohistochemical results.
e. Interpretation and grading of the immunohistochemical stains and statistical analyses. Months 33- 36.
RhoC, ER, PR and Her-2/neu stains have been evaluated and analyzed in conjunction with Task 1. We are in the process of interpreting the immunohistochemical results for WISP3 staining in this group as well. Once this is performed, we will analyze the value of RhoC and WISP3 expression in predicting response to therapy in this group of tumors.

Task 3. To study in detail the *in vivo* effect of WISP3 loss in modulating the response of invasive breast carcinomas with RhoC-GTPase over-expression to farnesyl transferase inhibitors. Months 24-36.

a. Prepare a panel of cell lines (SUM149 wt, SUM149/WISP3, HME/RhoC, SUM185 wt and MCF10AT wt). Since all these cell lines have been prepared in our preliminary work, getting them ready for injection with take approximately 3 weeks. Month 25-26.

b. In vivo mice experiment (injection of cell lines, tumor development and treatment with farnesyl transferase inhibitor). Months 27-30.

c. Histological and immunohistochemical study of the excised tumors stained with anti-RhoC and anti-WISP3 antibodies. Months 30-32.

d. Analysis of the immunostains. Months 32-34.

e. Statistical analyses. Months 34-36.

We have not yet initiated the experiments in Task 3. They will commence this year.

In addition to the Tasks, we have performed seminal work in understanding WISP3 function, and how WISP3 and RhoC may cooperate to determine a highly aggressive inflammatory breast cancer phenotype, which we have published (Kleer et al, WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer. Breast Cancer Res. 2004;6(1):R110-5). In this work, we found that antisense inhibition of WISP3 in HME cells increased RhoC mRNA and resulted in an increase in cellular proliferation, anchorage independent growth and VEGF levels in the conditioned media. Conversely, restoration of WISP3 expression in the highly malignant IBC cell line, SUM149, was able to decrease the expression of RhoC protein.

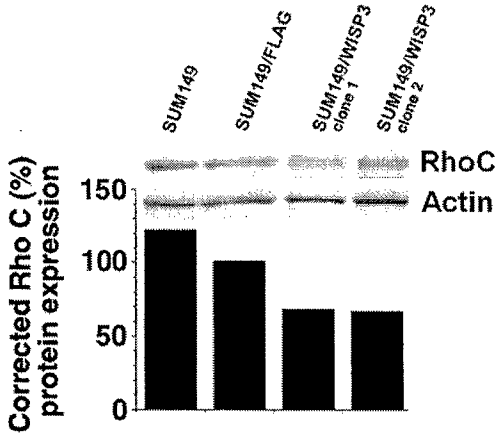


Figure 5. **Restoration of WISP3 expression in SUM149 inflammatory breast cancer cells decreases RhoC protein expression.** Western immunoblot of cell culture of SUM 149 cells, empty vector control (SUM149/ Flag), and two WISP3 expressing clones using antibodies for RhoC and actin. Gels were scanned and pixel intensity values were obtained. Values for RhoC were corrected for loading by dividing the RhoC pixel intensity by the actin pixel intensity.

In summary, WISP3 modulates RhoC expression in HME cells and in the IBC cell line SUM149. This provides further evidence in support that these two genes act in concert to give rise to the highly aggressive IBC phenotype. We propose a model of this interaction as a starting point for further investigations. This manuscript is included in the appendix.

We have also made an important contribution by elucidating that WISP3 is a secreted protein and that it modulates IGF signaling. This work is seminal, as no other tumor suppressor gene has ever been defined specifically for Inflammatory Breast Cancer. Previoulsy, we have demonstrated that WISP3 has tumor suppressor functions in IBC (Kleer et al, *Oncogene*, 21, 3172-3180, 2002), and we have gained insight into WISP3 as a modulator of IGF signaling. This work was presented at the AACR meeting in Washington DC, July, 2003 as an oral presentation, and has been recently published in *Neoplasia* and is included in the appendix. (Kleer et al, Neoplasia 2004 Mar-Apr;6(2):179-85).

In this work, we found that WISP3 is secreted into the conditioned media and into the lumens of normal breast ducts. Once secreted, WISP3 was able to decrease, directly or through induction of other

molecule(s), the IGF-1-induced activation of the IGF-IR, and two of its main downstream signaling molecules, IRS1 and ERK-1/2 in SUM149 IBC cells. Furthermore, WISP3 containing conditioned media decreased the growth rate of SUM149 cells. This work sheds light into the mechanism of WISP3 function by demonstrating that it is secreted, and that once in the extracellular media it induces a series of molecular events that lead to modulation of IGF-IR signaling pathways and cellular growth in IBC cells.

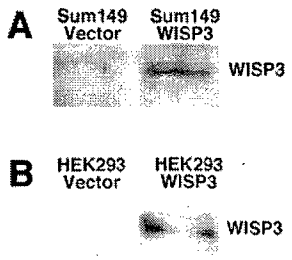


Figure 6. **WISP3 protein is secreted and detected in the conditioned media. A** Western immunoblot using anti-WISP3 polyclonal antibody detects WISP3 protein in the conditioned media of SUM149 cells transfected with WISP3 full-length cDNA. **B** Western immunoblot of the conditioned media of HEK-293 cells transfected with WISP3, detected using an anti-HIS antibody. WISP3 is not detected in the conditioned media of the control cells.

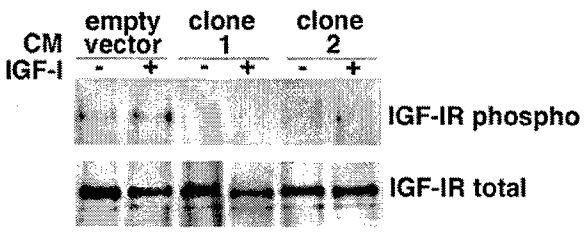


Figure 7. **WISP3 decreases IGF-I-induced phosphorylation of the IGF-IR.** Western blot of SUM149 cell lines bathed in WISP3+ and control (WISP3-) conditioned media. The experiment was carried out under baseline conditions (without IGF-I) and after stimulation with 20 ng/ml of IGF-I. The IGF-IR was precipitated from 500 μg of protein lysate with an anti-IGF-IR mAb and subsequently detected by immunoblot with an anti-IGF-IR β subunit polyclonal Ab. Tyrosine phosphorylation of immunoprecipitated IGF-IR was assessed with an anti-phosphotyrosine mAb PY20.

We have also established a stable siRNA inhibition of WISP3 expression in human mammary epithelial cells (HME) and characterized its functions. We found that WISP3 inhibition resulted in epithelial to mesenchymal transition of HME cells and induced motility and invasion. Moreover, these cells were more sensitive to the growth and proliferative effects of IGF-1 in the medium. These experiments suggest that WISP3 may be a key a regulator of IGF-1 effects in HME cells. We have recently submitted this paper for review to Breast Cancer Research.

We have also identified that EZH2 is a marker of aggressive breast cancer and that it promotes the neoplastic transformation of human mammary epithelial cells (Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt, RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA and Chinnaiyan AM. EZH2 is a Marker of Aggressive Breast Cancer and Promotes Neoplastic Transformation of Breast Epithelial Cells. *Proceedings of the National Academy of Sciences*, 100(20): 11606-11, 2003). For this study, we used the tissue microarrays constructed and stained them using a polyclonal antibody for EZH2, a transcriptional repressor. We found that EZH2 expression was an independent factor that predicts death from breast cancer. We have included a copy reprint of this paper in the appendix section.

In summary, this Award helped us complete several manuscripts dealing with key aspects of WISP3 and RhoC expression in breast cancer. We have developed key reagents and resources that will enable us to move forward in testing their clinical usefulness. We have also completed a major effort in understanding the function of WISP3 gene as it contributes to the inflammatory breast cancer phenotype.

Key Research Accomplishments

- Constructed four high density tissue microarrays
- Developed a relational database with the patient information
- Generated and tested a polyclonal antibody against WISP3
- Validation of RhoC as a novel tissue biomarker that predicts local recurrence and survival in patients with breast cancer.
- Investigated the mechanisms of cooperation between RhoC and WISP3 in determining the inflammatory breast cancer phenotype.
- Elucidated that WISP3 is a secreted protein and that it modulates IGF-I signaling cascade in inflammatory breast cancer
- Discovered that WISP3 inhibition in HME cells leads to epithelial to mesenchymal transition, and triggers invasion and motility.
- Discovered that EZH2 is a marker of aggressive breast cancer and that it promotes neoplastic transformation of mammary epithelial cells.

Training component of the Award

During this year, the P.I. has had a significant learning opportunity was to direct and work closely with the statistician to perform survival analyses for RhoC and for EZH2. In the laboratory, the P.I. learned how to design hairpin siRNA and develop stable cell lines lacking WISP3 expression. She learned how to interpret the experiments conducted that revealed that loss of WISP3 results in epithelial to mesenchymal transition and down regulation of E-cadherin. The P.I. attended and presented data at the annual meeting of the United States and Canadian Academy of Pathology in Vancouver, at the San Antonio Breast Cancer Symposium, and at the Era of hope Meeting in Philadelphia, where she had a poster presentation. She presented at the Breast Care Educational Forum (as well as attended this forum every Wednesday at noon). She also participated in the monthly meetings of the University of Michigan Breast Oncology Program to discuss research projects.

Reportable Outcomes

We are in a position to report that RhoC over expression is an early marker of aggressive breast cancers, even when they are small, and that it is a promising marker of prognosis and local recurrence in patients with breast cancer.

We can state that WISP3 is able to ameliorate the highly malignant features of inflammatory breast cancer. Specifically, WISP3 has growth and angiogenic inhibitory functions, at least in part though modulating IGF-receptor signaling pathways.

We can state that EZH2 is a marker of aggressive breast cancer, and that it can predict prognosis.

Research Manuscripts published for the period 2004- 2005:

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Ray ME, Yang ZQ, Albertson D, Kleer CG, Washburn JG, Macoska JA, Ethier SP. Genomic and Expression Analysis of the 8p11-12 Amplicon in Human Breast Cancer Cell Lines. *Cancer Research* 64(1):40-47, 2004.

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Schott AF, Roubidoux MA, Helvie MA, Hayes DF, Kleer CG, Newman LA, Pierce LJ, Griffith KA, Murray S, Hunt KA, Paramagul C, Baker LH. Clinical and Radiologic Assessments to Predict Breast Cancer Pathologic Complete Response to Neoadjuvant Chemotherapy. *Breast Cancer Research and Treatment*, In Press.

Ben-David MA, Kleer CG., Paramagul C, Griffith KA, and Pierce LJ. Is LCIS a Component of Breast Cancer a Risk Factor For Local Failure Following Breast- Conserving Therapy? Results of a Matched Pair Analysis. *Cancer*, In Press.

Pan Q, Bao LW, Kleer CG, Sabel M., Merajver SD Protein kinase Cε is elevated in high grade breast cancer and a novel target for RNA interference anticancer therapy. *Cancer Research*, In Press.

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Koker M, and Kleer CG. Smooth muscle actin and p63 in the diagnosis of difficult lesions of the breast. Poster Presentation, USCAP Meeting, Vancouver, BC, 2004.

Koker, M., Griffith K, Newman L, Sabel M, Rubin MA and Kleer CG. Pathologic Factors Predictive of Sentinel Lymph Node Metastasis in Patients with Breast Cancer. Poster Presentation, USCAP Meeting, Vancouver, BC, 2004.

Witniewski A, Rubin MA and Kleer CG. AMACR Expression in Breast Adenocarcinomas. Poster Presentation, USCAP Meeting, Vancouver, BC, 2004.

Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA and Chinnaiyan AM. EZH2 is a Marker of Aggressive Breast Cancer and Promotes Neoplastic Transformation of Breast Epithelial Cells. Platform Presentation, USCAP Meeting, Vancouver, BC, 2004.

Kleer CG, Griffith K, Sabel S, Gallagher G, Merajver SD. RhoC GTPase is a New Tissue Biomarker Predictive of Local Recurrence in Patients with Breast Cancer. Poster Presentation, AACR meeting, Orlando, FL, 2004.

Kleer CG, Griffith K, Sabel MS, vanGolen KL, Gallagher G, Wu, ZF, Merajver SD. RhoC-GTPase is a Novel Tissue Biomarker Associated with Biologically Aggressive Carcinomas of the Breast. Poster Discussion Session, San Antonio Breast Cancer Symposium. December 8-11, 2004.

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Conclusion

We are encouraged by our progress. We want to move forward and test the clinical utility of WISP3 and in combination with RhoC and other markers, in detecting aggressive breast cancer phenotypes before they develop metastases. We also wish to explore the relationship between WISP3 and the IGF-receptor pathway in more depth. These are the directions we are moving on for this year.

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